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NONSYMBIOTIC GERMINATION OF ORCHID SEEDS

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(WITH THREE FIGURES)

The germination of orchid seeds for a long time has been recognized as difficult and generally uncertain of attainment. Practical orchid growers for years have attempted to find a method which will insure germination. They meet with success at times, but fail utterly on a second attempt with the same method. Moreover, two sowings made at the same time and under apparently identical conditions may result in germination in the one case and failure in the other. There are growers in England, France, and also the United States who, if one may believe reports, are consistently successful in germinating the seeds of the commercially important orchids. The grower, however, is naturally unwilling to part with the details of his method. From the scientific aspect it is doubtful whether he can explain the cause of his success. Generally speaking it may be stated that practical orchid growers have not yet solved the problem of producing orchid plants from seeds.

The difficulty of germinating seeds of orchids is due in part to inherent causes, but undoubtedly is due also to environmental factors. The extremely small size of the embryo renders it liable to death if it becomes desiccated. Generally the seeds are sown on a substratum rich in organic matter, such as sawdust, leaf mold, wood or bark, peat, sphagnum, or mixtures of the two last-named substances. These substances are favorable for the growth

of fungi and algae, and the embryos may be killed because of being covered by these organisms, or more likely by injurious substances produced by the decomposition of these organisms. The work of BURGEFF (4) and BERNARD (2) demonstrates that death may be due to pathogenic fungi, and the writer's experiments in transplanting seedlings from tubes to open pots demonstrate clearly this danger. In addition to these factors, attention must be given to preventing loss due to insect pests. As suggested, however, there are apparently inherent characteristics of the seeds which make for refractory germination. It is this which attracted the attention of BERNARD, who in a number of publications presented evidence tending to show that the germination of the seeds and the subsequent growth of the seedlings are dependent upon infection by certain strains of the fungus which generally is found living in the orchid root, and which BERNARD considered to be *Rhizoctonia*. BURGEFF came to substantially the same conclusions, maintaining that germination was possible only when the embryo became infected with the proper strain of the fungus, to which he gave the name *Orcheomyces*, without attempting to classify it.

BERNARD and BURGEFF both pointed out that infection of the embryo began at the suspensor end of the seed, and that in the case of *Cattleya* and related forms the primary infection occurred through the delicate suspensor. Growth occurred if only the lower portion of the embryo became infected, and if the infection continued beyond approximately the lower third of the embryo, then death of the embryo resulted. It was also observed in germinating embryos that the fungus disintegrated in the infected zone, forming clumps of disintegrated hyphal material in the cells similar to the clumps found in cells of the root. It was the opinion of BERNARD that the fungus was digested by the orchid embryo. The essential point to be noted, however, is that a delicate balance between the host and the fungus apparently must be maintained in order to insure germination and also to prevent death of the embryo.

Granting for the present that a symbiotic relationship exists between the fungus and the embryo, it is nevertheless true that failure of germination is more common than success, even when the fungus is provided. BERNARD'S experiments reveal case after

case in which the introduction of the fungus was followed by death of the seeds or failure to germinate. He states as follows:

> The germination by inoculation is not obtained without certain difficulties. For five years I have sown seeds of diverse species of orchids in culture tubes, each of which contained 100 seeds, and these I have inoculated with *Rhizoctonia* obtained from the roots. Altogether, I have obtained a few hundreds of seedlings, but I underestimate when I place the number of seeds used in my experiments at 50,000. For the majority of the seeds, the association with the fungus that I have placed in their presence has been merely passive and without effect, or impossible or rapidly injurious to the embryos.

The explanation generally offered in these cases is that "activity" of the fungus was altered or the proper strain was not employed, so that the essentially delicate balance between the fungus and the embryo was not maintained.

In certain experiments BERNARD succeeded in germinating seeds of *Cattleya* and *Laelia* without the intervention of the fungus. This was accomplished by using a more concentrated solution of salep. Salep (KING, 6) is the dry powder obtained by pulverizing tubers of certain orchids, and contains, principally, mucilage 48 per cent, starch 27 per cent, and proteins 5 per cent. It probably contains also some sugar as well as soluble mineral matter. The seedlings obtained in this way were in every respect normal and the germination was very regular. BERNARD suggests that some such method might be developed for practical purposes, since the results with the fungus are so unsatisfactory.

The increasing importance of orchid culture in America, the difficulties in and the restrictions on the importation of orchid plants, and the desirability of creating new hybrid forms, make particularly desirable a method for germinating the seeds. Certain data from the experiments of BERNARD and BURGEFF, indicating that soluble organic compounds might cause germination, and my own previous experiments (7) on the organic nutrition of plants, demonstrating that various sugars have a very favorable influence on growth, are indications that germination of orchid seeds might be obtained by the use of certain sugars. This proved to be true.

The results here reported describe a method for germinating the seeds under sterile conditions, the influence of certain sugars

on growth of the embryos, the influence of different concentrations of sugar on growth, the effects induced by certain plant extracts, the favorable influence of certain bacteria, and experiments on transplanting. In the discussion are treated critically the ideas expressed by BERNARD and BURGEFF with respect to the function of the fungus.

For a clearer understanding of the data that follow, it is desirable to trace briefly the mode of development in the germination of seeds of *Cattleya* and *Laelia*. For a detailed discussion, BERNARD'S paper should be consulted. The embryo is somewhat oval-shaped, and is undifferentiated except that the cells at the basal region are large, while those at the apical region are smaller. This is the meristematic region. At the base is subtended a delicate suspensor. The embryo is inclosed within a transparent integument with an opening at the lower end through which the suspensor may protrude. The maximum length of the embryo of *Cattleya* or *Laelia* is about 250μ and the width about 75μ.

Germination consists, first, in an enlargement of the embryo in a transverse direction until a small spherule stage is reached. Accompanying this development there is the formation of chlorophyll, generally more pronounced in the meristem region. The embryo when it ruptures the integument has a width of about 175μ and a length of about 270μ. At the time of rupturing the integument, absorbing hairs begin to grow out from the epidermis. Subsequent development consists in a further enlargement of the embryo, other absorbing hairs begin to develop near the basal region, and there is attained a large spherule or top-shaped structure characterized by a marked depression at the upper surface. Following this there appears in the middle of the depression the first leaf point, which subsequently develops into the first leaf. During this period there is a continued increase in the diameter of the embryo, so that a disklike structure is formed which has been termed by BERNARD the protocorm. At the meristematic region a second and a third leaf may unfold, elongation may occur, and a distinct stem is apparent. The first root may arise either from the protocorm or from the stem below the second or the third leaf. The period required for these developments is generally

from four to six months. Under greenhouse conditions this advanced stage has apparently been attained in some cases in a shorter time.

Methods

Unless otherwise indicated, all cultures were made using agar slopes in culture tubes 180 mm. × 18 mm. The nutrient solution used was either Pfeffer's or a modification referred to hereafter as solution B. The solutions were made up as follows:

Solution B	Pfeffer's
$Ca(NO_3)_2$, 1 gm.	$Ca(NO_3)_2$, 4 gm.
K_2HPO_4, 0.25 gm.	K_2HPO_4, 1 gm.
$MgSO_47H_2O$, 0.25 gm.	$MgSO_47H_2O$, 1 gm.
$Fe_2(PO_4)_3$, 0.05 gm.	KNO_3, 1 gm.
$(NH_4)_2SO_4$, 0.50 gm.	KCl, 0.5 gm.
Distilled H_2O, 1 l.	$FeCl_3$, 40 mgm.
	Distilled H_2O, 5 l.

Solution B was used because Burgeff stated that the orchid seeds utilized ammonium sulphate to better advantage than the nitrate salt. My own experience is not in accordance with this.

Generally 1.50 per cent agar was used, and all media and vessels were autoclaved at fifteen pounds pressure for thirty minutes. To prevent the lodging of spores and microorganisms on the cotton stopper of the culture tube, it was capped with a small vial which, fitting tightly over the cotton plug, inclosed the upper third of the tube. The use of the vial cap was essential because otherwise, under the moist greenhouse conditions, contamination resulted from spores growing down through the cotton plug or between the plug and the tube. By using the vial cap cultures remained pure even after a year in the greenhouse.

The cultures were all grown under aseptic conditions. For sterilizing the seeds, the calcium hypochlorite method of Wilson (13) was used. For this purpose 10 gm. of calcium hypochlorite was added to 140 cc. of distilled water. This was vigorously shaken for a few minutes and then filtered. The clear filtrate was used for sterilizing the seeds. The quantity of seeds desired was placed in a small test tube and the clear filtrate added. The tube was then shaken until each seed became moistened with the solution.

This was repeated several times, since the seeds generally float together in a mass at the surface of the liquid. The period of exposure was about fifteen minutes, although in some preliminary experiments with seeds of *Cattleya* and *Laelia* no injury was noted after a three hours' exposure. The seeds were transferred from the sterilizing solution, without any previous rinsing in water, by the use of a platinum needle. With the small loop used, it was possible to pick up about 100 seeds. These were scattered over the surface of the agar slope. The cultures were maintained in moist chambers in the greenhouse shaded by cheesecloth from direct sunlight, with the temperature between 15° and 35° C. In determining growth, the embryos were measured by means of an ocular micrometer. As was shown by both BERNARD and BURGEFF, the width of the embryo or the protocorm may be accepted as a good criterion of the degree of growth. Other data are included, such as percentage of germination, time of formation of first leaf, color, starch content, etc.

Preliminary experiments

EXPERIMENT 1.—On December 7, 1918, seeds of *Cattleya Schroederae*×*C. gigas* were sterilized by treating them for two hours with the calcium hypochlorite solution. The seeds were sown on agar slopes. The medium used in one case was an extract of peat, made by autoclaving 300 gm. of bog peat, such as is used for potting orchids, with 1200 cc. of tap water. This was filtered and the clear brownish filtrate used. The other medium was made by autoclaving 400 gm. of dormant canna tubers with 600 cc. of water for thirty minutes. By January 7, 1918, the seeds on both were in the small spherule stage and were green. On April 10, four months after planting, the seeds on the canna medium had germinated, the seedlings having one and two leaves. On the peat agar medium the embryos were a little larger than on January 7, 1919, but not significantly different.

EXPERIMENT 2.—The media used were extracts of carrot and garden beet. The carrot extract was made by autoclaving 70 gm. of young carrots (root) with 75 cc. of tap water, and the garden beet extract was made by autoclaving 50 gm. of young beets (root)

with 75 cc. of water. The extracts were filtered, and to the clear filtrate 1.25 per cent agar was added. Seeds of *Cattleya labiata* × *C. aurea* were sterilized and planted on February 14, 1919. On May 13 some of the seeds in each had germinated and the remainder were almost germinated, that is, they were just at the point of producing the first leaf.

EXPERIMENT 3.—The media used were Pfeffer's alone and Pfeffer's plus 1 per cent sucrose. Seeds of *Cattleya mossiae* were planted on January 14, 1919. On July 1 the seeds in the sucrose culture had germinated, one leaf showing. On the Pfeffer's alone the embryos were in a small green spherule stage, the diameter being about 250μ, while the diameter of the embryos on sucrose was about 1000μ.

EXPERIMENT 4.—Seeds of *Cattleya intermedia* × *C. Lawrenceana* were sown on July 18, 1919, on solution B plus 2 per cent glucose on the one hand and 2 per cent sucrose on the other. Owing to an absence from the University, the cultures were not examined until June 9, 1920. At that time, in both glucose and sucrose cultures, the seedlings were well developed, although the culture media had lost most of the water by evaporation. The seedlings had two or three leaves and one or two roots, some of the roots being 4 mm. in length.

Influence of certain sugars and plant extracts on germination

The preliminary experiments show that germination of seeds of *Cattleya* and *Laelia* is possible without the aid of the fungus, provided soluble organic substances are present, particularly sugars. In all these cases the leaf point appeared only after three months, and yet under practical greenhouse conditions, when the seeds are sown merely on a compost of peat and sphagnum or other organic material, the leaf points may appear in a shorter time. For example, according to Mr. T. L. MEAD, of Oviedo, Florida, seeds of *Cattleya* have shown leaf points in as short a period as thirty-five days. Some of the media used by him were oak bark, magnolia bark, and a compost of decayed leaves and sphagnum.

It is possible, of course, that under these practical conditions the fungus is a factor in the growth. Is it possible also that certain

of the products produced on decomposition of the organic substances, such as the auximones described by BOTTOMLEY (3) or the vitamine water-soluble B, are involved in the germination of orchid seeds? Of course other factors may be involved, such as the hydrogen ion concentration, mineral salts, or the rate of transpiration, particularly as influencing the organic composition of the plant.

That a full nutrient medium plus sugar is not capable of sustaining continued growth of higher plants was shown by KNUDSON and LINDSTROM (8) in their experiments with albino corn. The plants kept either in the light or in the dark and supplied with one of several different sugars all died after a month or two. These experiments, together with the work of BOTTOMLEY on auximones, the beneficial influence of vegetable extracts on the growth of fungi recently described by DUGGAR (5) and by WILLAMAN (11), and the beneficial influence of vegetable extracts on the growth of yeast as described by WILLIAMS (12) and by BACHMANN (1) suggest that more rapid germination and more vigorous plants could be obtained if a vegetable extract was added to the nutrient medium.

With no idea of determining what specific substances are involved in stimulating growth, but in the endeavor to develop a rapid and effective method for the germination of seeds of certain orchids, the experiments described in table I were made. The nutrient solution used was solution B.

The extracts used were prepared as follows. Potato extract: 200 gm. of new potato with the skin removed, with 300 cc. of distilled water; wheat extract: 200 gm. of air-dried soft wheat, with 300 cc. of distilled water; beet extract: 200 gm. of a red garden beet cut into small pieces, with 200 cc. of distilled water. Extraction was made by autoclaving for fifteen minutes at fifteen pounds pressure, and the extracts obtained by filtration. The yeast juice was obtained as follows: three four-liter flasks, each containing three liters of WILLIAMS' solutions, were inoculated with a cake of Fleischman's yeast, and after a week the yeast was filtered from the solutions, autolyzed at 37° for twenty-four hours, and then dried by suction and washing with ether. Seventy gm. of yeast was then steamed for ten minutes with 250 cc. of distilled

water. The liquid was filtered and made up to a liter volume by the addition of distilled water.

All cultures were made in quadruplicate. The individual cultures of each series were strikingly uniform in growth, so that

TABLE I

Laelia-Cattleya HYBRID NO. 1;* SEEDS PLANTED AUGUST 31, 1920; MEASUREMENTS MADE JANUARY 27, 1921

CULTURE NO.	CULTURE SOLUTION	WIDTH OF EMBRYO IN MICRONS			PERCENTAGE OF GERMINATION	ORDER OF SUPERIORITY, MARCH 27
		Minimum	Maximum	Average		
B 53...	Full nutrient 50 cc.+50 cc. beet extract	242	582	407	0	5
B 17...	Full nutrient 50 cc.+50 cc. potato extract	339	630	459	0	5
B 60...	Full nutrient 50 cc.+50 cc. wheat extract	174	436	291	0	7
B 39...	Full nutrient+2% glucose	485	1261	970	20	4
B 28...	Full nutrient solution alone	194	242	213	0	8
B 27...	Full nutrient+2% glucose 90 cc.+10 cc. beet extract	485	1358	814	70	3
B 34...	Full nutrient +2% glucose 90 cc.+10 cc. wheat extract	582	1358	979	90	1
B 55...	Full nutrient+2% glucose 98 cc.+2 cc. yeast extract	582	1552	1076	90	1
B 66...	Full nutrient+2% fructose	776	1260	940	60	1
B 48...	Full nutrient+2% fructose 90 cc.+10 cc. beet extract	485	1358	902	80	2
B 22...	Full nutrient+2% fructose 90 cc.+10 cc. potato extract	485	1202	1008	90	1
B 31...	Full nutrient+2% fructose 90 cc.+10 cc. wheat extract	582	1260	902	70	2
B 5...	Full nutrient+2% fructose 98 cc.+2 cc. yeast extract	582	1358	1047	80	1
B 43...	Full nutrient+2% fructose 96 cc.+4 cc. yeast extract	582	1746	970	60	2
B 14...	Beet extract alone	242	872	388	0	5
B 69...	Potato extract alone	339	679	504	0	5
B 6...	Wheat extract alone	242	436	358	0	6
B 74...	Yeast extract alone	87	339	194	0	7

* Composition: *L. Perrinii* Lindl. ¼; *C. labiata* Lindl. ¼; *C. amethystoglossa* Reichb. ¼; *C. intermedia* Grah. ¼.

for the first measurements only one culture for each series was taken, and forty individual measurements were made for each culture. The measurements given in table I were made on January 27,1921. The order of superiority of the cultures on March 27 is recorded in the last column of the table. Similar data, not

included, were obtained in a like experiment with *Laelia-Cattleya* hybrid no. 2.

The degree of development represented by the numbers 1 to 8 is as follows: (1) dark green seedlings, most of these with two leaves and a few showing roots; (2) seedlings the same as no. 1, but light green; (3) seedlings light green, most of them with one leaf and a few showing two leaves; (4) seedlings light green, with only one leaf and that leaf short; (5) about 50 per cent of embryos showing leaf point; (6) embryos just showing a depression in meristem region; (7) advanced spherule stage; (8) smaller spherule stage.

The data in table I show that fructose is more favorable for growth of the embryos than glucose. This is apparent not only in the percentage showing leaves, but in the general appearance of the cultures. The embryos in the glucose cultures were whitish or yellowish in color. On the other hand, the fructose cultures were dark green. A more striking difference was noted on March 27, when in the glucose cultures the embryos were still yellowish and had shown no appreciable gain since January 27. The fructose cultures, on the other hand, had progressed and were still more markedly superior to the glucose cultures than on January 27. Fig. 1 shows the fructose culture and the nutrient solution culture minus sugar.

The addition of a plant extract to the glucose cultures has a marked effect on growth and chlorophyll development. In each case the percentage of germination is higher than with glucose alone, and the ranking of glucose-containing cultures on March 27 indicates that those with yeast or wheat extract rank with the best cultures, that with beet extract ranks in the third group, and the cultures with glucose alone fall in the fourth group. The addition of plant extract to the fructose-containing media is practically without any beneficial effect.

The loss or lack of development when glucose is supplied in the nutrient solution has been noted by MAZÉ and PERRIER (9) for corn, and by SERVETTAZ (10) in nutrition experiments with moss. In the case of orchid embryos the chlorophyll makes its appearance only when the leaf is developing, and then generally only in the leaf. Even then the leaves are only of a light green

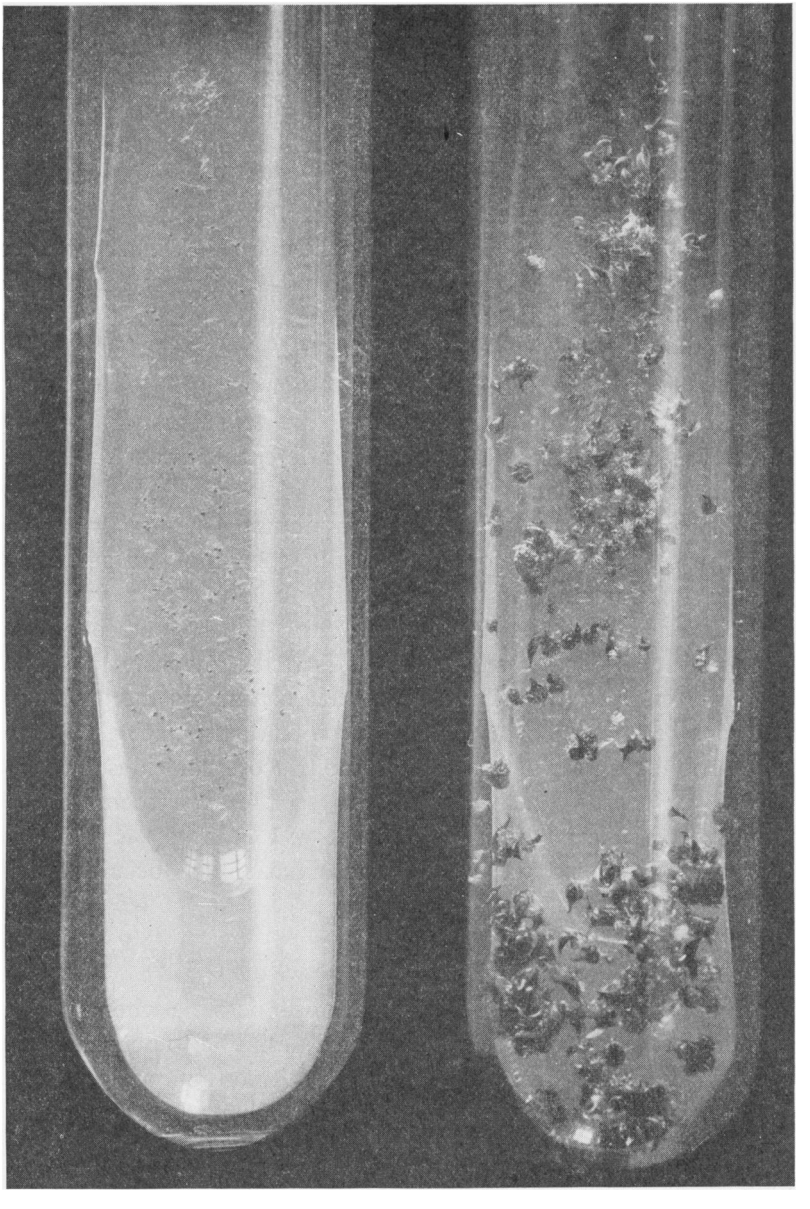

FIG. 1.—Solution B, embryos in small spherule stage; solution B+2 per cent fructose, seedling stage; ×2.

color. Is chlorosis due to a non-utilization of nitrogen or iron in the presence of glucose, or in the upbuilding of chlorophyll is glucose less favorable than fructose? Certain experiments now in progress may throw some light on this interesting point, and it is therefore desirable to await the results before speculating any further.

On January 27 the leaf point was not yet evident in any of the plant extract cultures. On March 10, however, in the wheat, beet, and potato extracts, embryos with leaf points were apparent, and a little later the same was noted for the yeast extract cultures. After several months more, seedlings with one and two leaves were to be noted in all these cultures.

Is germination on these extracts due to sugars or to other substances? Analyses made of the different extracts show that the potato, wheat, and yeast extracts had a sugar content of less than 0.025 per cent. The extracts diluted one-half with the nutrient solution, therefore, practically speaking had no sugar. The beet extract had a sugar content of 0.80 per cent, yet it did not permit any better germination than did the potato extract with merely a trace of sugar. As indicated previously, it should be borne in mind that the beet extract contains some substance injurious to the embryo. Furthermore, the stimulating effect of the plant extracts when added to the glucose solutions must be due to substances other than sugars. In the experiments on the influence of concentration of sugar, it will be noted that on the concentration of 0.05 per cent no germination has occurred even after four months.

Influence of concentration of sugar

In view of the fact that germination is possible when sugar is supplied in the culture media, it seemed desirable to determine the concentration most favorable for growth. Accordingly several series of experiments were made, a number of which are here reported.

In the first experiment seeds of *Laelia-Cattleya* hybrid no. 2 were used. These were planted November 12, 1920, and notes were made December 16, 1920, and January 11, February 15, and March 15, 1921. Each of the average figures given represents the average

of thirty separate measurements. The data are given in table II. The slow growth is well shown. There is in general a corresponding increase with increase in concentration, but the increase in concentration beyond 0.80 per cent is without any significant effect. On February 15 the embryos of all the cultures were examined for starch. It was found only in those cultures with 0.80 per cent glucose or higher. This fact is evidence that the absorption of glucose at a concentration of 0.80 per cent is in excess of the utilization, and consequently a higher concentration

TABLE II

INFLUENCE OF CONCENTRATION OF GLUCOSE, *Laelia-Cattleya* HYBRID NO. 2;* SEEDS SOWN NOVEMBER 12

CULTURE SOLUTION	AVERAGE WIDTH OF EMBRYOS IN MICRONS		
	December 16	January 11	March 15
Solution B	126	145	174
Solution B 0.05% glucose	184	232	247
Solution B 0.10% glucose	200	252	339
Solution B 0.20% glucose	242	281	475
Solution B 0.40% glucose	310	291	455
Solution B 0.80% glucose	291	339	533
Solution B 1.00% glucose	320	417	543
Solution B 2.00% glucose	320	436	523

* Composition: *C. Trianaei* Reichb. ½; *C. Loddigesii* Lindl. ¼; *L. purpurata* Lindl. ¼.

should be without any increased beneficial effect. It should be borne in mind that glucose used with solution B is not particularly suited for the germination of orchid seedlings, since there is induced constantly in the embryos a distinct chlorosis. It is probable that higher concentrations of sucrose or fructose would permit of a more rapid germination.

The results of several other experiments on the influence of different concentrations of glucose on the germination of seeds of *Cattleya* are in agreement with these results, and need no repetition. In an experiment with seeds of *Epidendron*, germination was obtained with a concentration of 0.2 per cent glucose. In the cultures with less than 0.1 per cent glucose, not only was there a less development of the embryos, but a large percentage of the seeds never showed any initial swelling and development of chlorophyll.

The detailed data are given in table III. The figures given under average width represent the averages of forty individual measurements. Only seeds that had shown an initial increase in diameter and were green were included.

In this experiment another interesting observation was made. Just previous to the formation of the leaf point, the embryos were gorged with starch. With the formation of the leaf, however, there was a disappearance of the starch, it having been converted into

TABLE III

INFLUENCE OF CONCENTRATION OF GLUCOSE, *Epidendron tampense*×*E. inosmun*; SEEDS PLANTED DECEMBER 8, 1920; NOTES TAKEN MARCH 17, 1921

CULTURE SOLUTION	CULTURE NO.	WIDTH OF EMBRYOS IN MICRONS			PERCENTAGE WITH LEAVES	REMARKS
		Minimum	Maximum	Average		
Full nutrient.....	P 2	116	203	145	.....	95% no change
Full nutrient+0.05% glucose	P 72	116	291	178	.....	90% no change
Full nutrient+0.1 % glucose	P 78	189	407	281	.....	80 %no change
Full nutrient+0.2 % glucose	P 81	339	630	465	10	30% no change
Full nutrient+0.4 % glucose	P 86	291	582	397	10	40% no change
Full nutrient+0.8 % glucose	P 91	194	582	446	20	50% no change
Full nutrient+1.00% glucose	P 99	339	582	446	10	30% no change
Full nutrient+2.00% glucose	P 104	291	630	446	10	25% no change

sugar, as evidenced by the fact that some of the embryos still showed a slight presence of dextrins.

Influence of microorganisms

Throughout the various experiments made a few cultures always became contaminated. Generally, if the contamination was a *Penicillium*, the embryos became covered by the mycelium and death resulted. Those embryos not covered often showed a marked increase in growth over the embryos in the corresponding uncontaminated cultures. The increase in growth may have been due to one or a combination of the following: an increase in the carbon dioxide content of the tube; a change in the chemical character of the nutrient medium, brought about either by secretion of organic substances from the fungus or by products produced on decomposition of the fungus; or changes in the sugar effected by extracellular enzyme action.

In an experiment with seeds of *Epidendron* growing on solution B plus 0.80 per cent glucose, one of the cultures became contaminated with a species of *Actinomyces*. The result was that when the embryos in the contaminated culture were dark green, with one and two leaves, the embryos in corresponding uncontaminated cultures were still white or yellowish and only one or two of them showing the leaf point. In the contaminated culture the embryo

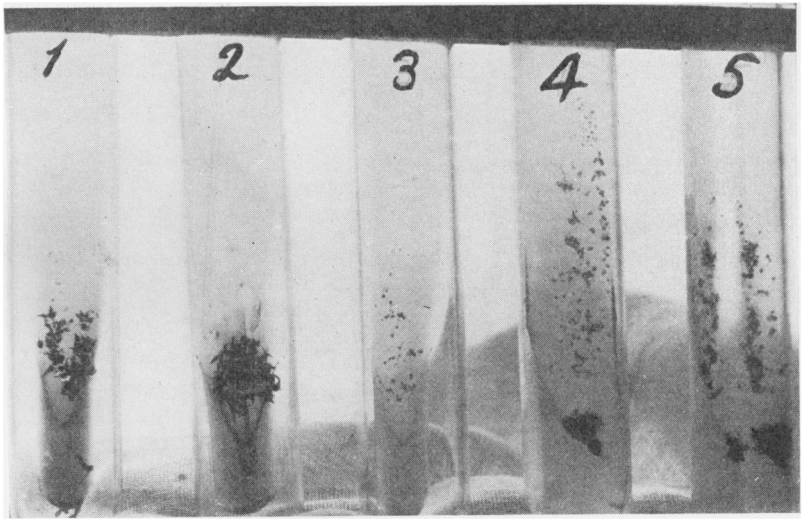

FIG. 2.—Culture no. 1, solution B+2 per cent glucose contaminated by *Actinomyces* sp. (corresponding check like no 3); culture no. 2, Pfeffer's +2 per cent sucrose inoculated with *Bacillus radicicola;* culture no. 3, same but not inoculated; culture no. 4, solution B+2 per cent glucose; culture no. 5, solution B+2 per cent glucose inoculated with *B. radicicola*; ×⅔.

had an average width of 975 μ, while in the uncontaminated cultures the average width of the embryos was 600 μ.

In view of these observations, and since BOTTOMLEY reported a beneficial effect on the growth of *Lemna* by the addition to the nutrient solution of an aqueous extract of *Azotobacter* or of *Bacillum radicicola*, an experiment was made to determine the influence of the latter organism on germination. Ten tubes were prepared with Pfeffer's solution plus 2 per cent sucrose; five were inoculated with *B. radicicola* from alfalfa, and five were left uninoculated. Seeds of *Epidendron* were sown on December 18, 1920. On March 5, 1921, 80 per cent of all the seeds in the inoculated tubes had

germinated and possessed one or two leaves. On the cultures not inoculated, the embryos lacked chlorophyll, and not one had produced even the leaf point. Most of the embryos exhibited the depression at the meristem region. Two months later some of the embryos, although still lacking chlorophyll, had formed the leaf point; while in the inoculated cultures the seedlings had two and three leaves and some had already produced roots (fig. 2).

The results appeared so unusual that the experiment was repeated with seeds of *Laelia-Cattleya* hybrid no. 3, using solution B and also Ashby's solution. The composition of the nutrient

TABLE IV

INFLUENCE OF *Bacillus radicicola* ON *Laelia-Cattleya* HYBRID NO. 3;* SEEDS SOWN MARCH 14, 1921; NOTES TAKEN AUGUST 10, 1921

Nutrient medium	Average width in microns
Solution B	271
Solution B+0.1% glucose	407
Solution B+1.0% glucose	814
Solution B+inoculated	194
Solution B+0.1 glucose inoculated	378
Solution B+1.0% glucose inoculated	834
Ashby's solution	194
Ashby's solution+0.1% glucose	446
Ashby's solution+1.0% glucose	698
Ashby's solution+inoculated	164
Ashby's solution+0.1% glucose inoculated	397
Ashby's solution+1.0% glucose inoculated	970

* Composition: *C. superba* Schomb. ¼; *C. Dormaniana* Reichb. ¼; *C. Warscewiczii* Reichb. ¼; *L. purpurata* Lindl. ¼.

solution and the width of embryos are given in table IV. The favorable influence of *Bacillus radicicola* was noted only in the cultures having 1 per cent glucose. On solution B+1 per cent glucose the diameters of the embryos averaged the same for both the inoculated and the uninoculated seeds, but there was a striking difference in the color and the number with leaves. In the uninoculated, 20 per cent of the embryos were showing the leaf point, but the embryos were whitish in color. In the inoculated, 50 per cent of the embryos showed leaves, the leaf development was greater, and the embryos were green.

On Ashby's medium plus 1 per cent glucose, the beneficial effect of the organism was more apparent. There was a marked increase in the width of the embryos. In the inoculated cultures all the embryos had produced one or two leaves and the embryos were dark green. In the uninoculated cultures only 25 per cent of the embryos had produced a leaf point and the embryos were chlorotic (fig. 2). On both solution B and Ashby's+1 per cent glucose, the influence of *Bacillus radicicola* was so strikingly beneficial that it was observable immediately.

In the cultures with 0.10 per cent glucose or no glucose, the influence of *B. radicicola* seemed to be injurious, for in all the inoculated cultures the average width of the embryos was less than in the uninoculated cultures.

The cause of this favorable influence of *Bacillus radicicola* on the growth of orchid embryos remains yet to be determined. Some experiments were also made in which the cultures were inoculated with *Azotobacter* sp. In every case, however, there was a marked retardation in growth.

Transplanting experiments

On July 12, seeds of *Laelia-Cattleya* hybrid no. 2 were sown on solution B plus 2 per cent fructose plus 1.5 per cent agar. As culture vessels, Erlenmeyer flasks of 150 cc. capacity were used, and 30 cc. of the medium was employed. On October 14 the embryos were just on the verge of producing the leaf point. They were then transferred to six Erlenmeyer flasks (D 18 to D 23) containing 50 cc. of nutrient media, as follows: Pfeffer's solution+2 per cent glucose+0.1 cc. carrot decoction.

On March 1, 1920, the seedlings in cultures D 18 to D 23 had two and three leaves with a pronounced protocorm, and from the protocorm one and two roots had grown out, the roots varying from 1 to 3 cm. in length; whereas in the corresponding tube cultures, four seedlings in a hundred had produced roots, and these roots were only 2 mm. and 3 mm. in length. On March 11, 1921, seedlings were transferred from cultures D 22 and D 23 to liter Erlenmeyer flasks containing 300 cc. of solution B plus or minus sugar. Eight cultures were made, four with 2.0 per cent sucrose

and four without sugar. On August 20, 1921, notes were made on these cultures. In the sucrose cultures the seedlings had made a marked development. The largest seedlings had four and five leaves, some of the leaves being 2 cm. in length; while the roots of these seedlings, two or three in number, were 2–5 cm. in length (fig. 3). In the cultures lacking sugar the growth was less striking, the leaves being 2–4 mm. and the roots 2–10 mm. in length.

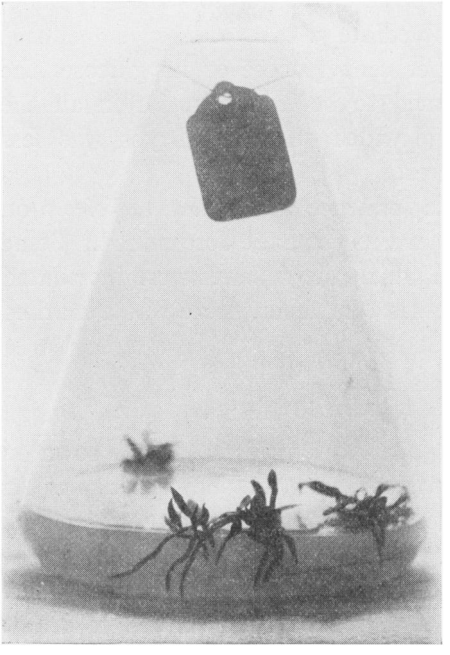

FIG. 3.—Seedlings one year old on solution B+2 per cent sucrose; $\times\frac{2}{3}$.

Seedlings were transplanted at the same time from D 23 to a compost of peat and sphagnum in ordinary flower pots. These seedlings, on August 20, 1921, showed leaves 5–7 mm. and roots 1–2 cm. in length. These seedlings were better than those planted on solution B in the liter flasks, but not so good as the seedlings on solution B plus 2 per cent sucrose in the liter flasks.

That better growth is possible in 150 cc. to 500 cc. Erlenmeyer flasks than in culture tubes was demonstrated repeatedly during these experiments. Erlenmeyer flasks of from 150 to 500 cc. capacity, containing the culture media left over after supplying the tubes with the requisite amount, were generally planted with the seeds that remained after the tubes were sown. In practically every instance germination took place sooner in the flasks than in the tubes. The probable explanation is that in the tube cultures the inward diffusion of carbon dioxide is impeded to a certain extent by the cotton plug, and, the volume of air in the tube being small, the carbon

dioxide is soon exhausted. In the larger flasks, however, the volume of air, and therefore the volume of CO_2, is much greater, from six to twenty-five times as great, and furthermore, the area through which the CO_2 can diffuse is greater by virtue of the larger mouths of the flasks. That the diffusion of CO_2 is impeded by a cotton stopper was shown in a previous paper (KNUDSON 7).

From the practical standpoint this would seem to be a method for the propagation of orchid seeds. The seeds may be germinated in the small culture tubes or in larger containers, and when roots are produced they may be transplanted either to pots in the open or transferred to sterile culture in larger flasks. My efforts to develop the seedlings on peat sphagnum mixture in flower pots in the open resulted in failure on several different occasions, due in one case to the temperature running up to 40° C., which permitted a pathogenic fungus to destroy utterly the seedlings in about twenty-five pots. In another case, during an absence from the city the seedlings were destroyed by insects. Previous to the misfortunes which the seedlings experienced they had been growing for periods of three and four months and were making satisfactory development. Other experiments are now in progress on this phase of the question. Some tubes were sent to Mr. T. L. MEAD, of Oviedo, Florida. Some of these were transplanted four and five months ago, and according to a recent communication from Mr. MEAD, the seedlings transferred are continuing growth, and but few seedlings were lost as a result of transplanting. The results of certain experiments now in progress indicate that more rapid growth will be obtained if the culture seedlings are transferred to sterile media containing sugar and grown for a year or two under these conditions. This method, moreover, has the advantage that the seedlings are not exposed to the depredations of insects or the ravages of parasitic fungi. Furthermore, contamination of the cultures by *Penicillium* or *Aspergillus* is without any injurious effect, provided that at the time of transplanting the seedlings have roots.

Discussion

What is the significance of these results in relation to the views advanced by BERNARD and BURGEFF, and quite generally believed

today, that for the germination of orchid seeds infection of the embryo by the appropriate fungus is essential? BERNARD believed that the action of the fungus was a physicochemical one, in that the fungus would cause an increase in a concentration of the cell sap, which increase in concentration would induce germination and the formation of a protocorm in somewhat the same way that the form of algae could change by increasing the concentration of the external solution. He points out that the fungus can invert sucrose and this may occur in the embryo.

The writer believes that the fungus may bring about germination in another way. As previously pointed out, in all his media BERNARD used a substance known as salep. This is a powder derived by grinding the dried tubers of certain species of orchids, and is rich in pentosans and starch, containing also about 5 per cent of organic nitrogenous substances. It probably contains some soluble organic and inorganic matter, judging from freezing-point determinations made by BERNARD. In view of the fact that organic matter is present, it is conceivable that the influence of the fungus might be to digest some of the starch, pentosans, and nitrogenous substances; which digestion products, together with secretions from the fungus or products produced on decomposition of the fungus, might be the cause of germination. In brief, it is conceivable that germination is induced not by any action of the fungus within the embryo, but by products produced externally on digestion or secreted by the fungus. Unfortunately I have not as yet succeeded in satisfactorily isolating the organism stated as necessary by BERNARD, nor has it been possible to purchase salep. Work is still in progress on this problem. There are, however, certain facts which support the idea that the action of the fungus is not necessarily internal. BERNARD does not give any analyses of the medium used, but he does give certain cryoscopic data. The medium generally used, made with salep, had a freezing-point depression of 0.01° C. Assuming that this depression ($\triangle$) is produced largely by hexose sugars, it would indicate at the outset of the experiment a concentration of hexose sugars equivalent to 0.1 per cent glucose. It is not possible, of course, to say that this depression is due entirely to hexose sugars; perhaps other sugars are present,

as well as other soluble organic and inorganic substances. The significant fact is that at the outset some soluble organic substances are present.

In addition to the soluble substances present, which apparently are not sufficient in quantity nor suitable as regards quality to permit of germination, there are to be considered the insoluble organic substances, pentosans, starch, and organic nitrogenous substances. Digestion of starch by the fungus would augment the concentration of sugar, and digestion of the organic nitrogenous substances might produce certain products which would make possible the germination of the seeds.

In my experiments it is true that the sugar used generally was of a relatively high concentration, but in the case of *Epidendron*, germination was obtained on 0.2 per cent glucose, which sugar is not particularly favorable for growth. That other substances besides sugar exert a pronounced influence is shown by the experiments on the beneficial effects of adding certain plant extracts to the glucose-containing solutions. The fact that other substances besides sugars may be important in the germination is shown by the experiments in which germination was obtained on decoctions of yeast, wheat grains, or of potato. All of these extracts contained less than 0.02 per cent total sugar. The experiments on the influence of *Bacillus radicicola* also lend weight to the idea that certain extraneous products may markedly influence germination.

BURGEFF, in certain of his experiments, used 2 per cent salep, but in other experiments he used starch, sucrose, or glucose. The explanations offered with respect to the function of the fungus in discussing BERNARD'S work may be used to account for the results obtained by BURGEFF. There may appear to be rather more difficulty in explaining the function of the fungus in the cultures containing either glucose or sucrose. It will be necessary to discuss these in more detail.

In one experiment, seeds of *Cattleya* were sown in a tube containing a nutrient solution plus 0.33 per cent sucrose. After three months the embryos were 0.4–0.5 mm. in width. Then, according to BURGEFF, they remained stationary. Cultures four months old, inoculated and maintained in the dark at 23° C., produced the first

leaf at the age of eight months. BURGEFF does not state that the uninoculated cultures were maintained under the same condition, but presumably they were. Granting that the uninoculated culture did not produce leaves, it is possible to explain the germination on the basis of the inversion of sucrose, which would yield approximately concentrations of both glucose and fructose molecularly equivalent to the original sucrose concentration. In addition there is to be considered the possible influence of products secreted by the fungus or produced on decomposition of the fungus.

The favorable influence of saprophytic fungi and bacteria demonstrated by my experiments is paralleled by certain cultures of BURGEFF. He transplanted four months' old seedlings to a mineral nutrient medium containing salep. Some of the cultures became contaminated with saprophytic organisms. The uncontaminated culture showed little growth, if any, after three months; while the culture contaminated by *Penicillium* made a marked growth, the leaves being 4 cm. in length. Another saprophytic fungus in another culture likewise caused a marked increase in growth, the leaves being about 9 mm. in length; and in a third culture contaminated by bacteria the seedlings were of similar character to those of the pure culture, but apparently darker green in color. All of these seedlings had produced vigorous roots. In the pure culture some of the seedlings had died. BURGEFF considers that the more favorable growth in the tube with fungus contamination was due to the development of an acid reaction in the medium. There is a possibility that increased carbon dioxide content and other products produced by the organism are partly responsible. It should be stated that the seedlings originally transferred to these tubes had previously been infected by the essential fungus.

Another experiment of BURGEFF lends weight, however, to the idea that the fungus is effective in inducing germination as a result of certain reactions brought about within the embryo. In this experiment the culture medium consisted of a weak nutrient solution plus $\frac{1}{20}$ per cent starch. Seeds of a *Laelia-Cattleya* cross were planted, and the root fungus from each of seventeen different orchids was tested for its ability to induce germination. In the cultures uninoculated the embryos attained a width of 0.45 mm. in four months. In certain cultures only the suspensor became

infected. The diameter of the embryos in this case reached from 0.6 to 0.8 mm. in the same time. With infection still more advanced, but less than normal, a few seeds had leaves after seven months. Normally infected embryos produced leaves, and embryos that adhered to the wall of the tube likewise germinated. An infection more advanced than normal caused the same development as an infection slightly less than normal. When from one-half to two-thirds of the embryos became invaded, growth was less than in the embryos not infected, and in another case the seeds were killed outright.

As stated, certain facts from these experiments make it difficult to explain the action of the fungus as purely external. If so, why should the fungi behave so differently in inducing or retarding germination? Unfortunately BURGEFF gives no details so that one may judge whether or not these results could be duplicated on second trial. Another difficulty is an adequate explanation for the germination of seeds adhering to the inner surface of the culture tube. It is possible, of course, that decomposition products of the fungus growing on the surface of the tube may have been the cause. It is desirable to await experiments with the fungus before attempting to discuss these points further.

There are other phases of the problem presented by BERNARD, especially the loss by the fungus of its capacity to induce germination after prolonged culture in the laboratory. It is entirely possible that there has been no loss in the fungus, but that at the time of inoculating the culture the physiological state of the embryos was such as to resist or permit of infection. Those in which the infection was confined to the lower cell could still germinate despite the fungus. Those invaded to a greater extent would be killed. These and other experiments of BERNARD and BURGEFF suggest that one of the causes for the failure of germination is the parasitic character of the fungus. In other words, it is possible that the fungus, instead of being an aid in normal germination, is a factor in the death of the embryos and consequently in the failure of germination.

In conclusion, it may be stated that the evidence for the necessity of the fungus for germination has not yet been conclusively proved. The evidence is conclusive that under conditions of pure

culture employed by both BERNARD and BURGEFF germination of the seeds is dependent on the fungus. There is still considerable work to be done, however, before the validity of the fungus hypothesis can be proved or disproved.

Summary

1. A method is given for sterilizing seeds of certain orchids and for growing them under sterile conditions.

2. Germination of seeds of *Laelia*, *Cattleya*, and related forms is possible without the aid of any fungus when certain sugars are supplied.

3. Fructose appears more favorable than glucose.

4. In the presence of glucose, chlorosis of the embryo generally results.

5. Germination is possible on certain plant extracts containing merely traces of sugar.

6. Embryos in sugar-containing cultures accumulate a considerable reserve of starch.

7. The concentration of glucose is important in the growth of the embryo.

8. *Bacillus radicicola* from alfalfa and certain other microorganisms on certain media have a favorable influence on the development of chlorophyll and germination.

9. Seedlings have been transplanted from tubes to large flasks and growth has continued.

10. The results thus far obtained indicate that the method is of value in the propagation of orchids from seeds.

11. The idea is advanced that the necessity of fungus infection for germination has not yet been proved.

12. One cause of failure of germination may be the pathogenic character of some of the endophytic fungi.

Mr. T. L. MEAD, of Oviedo, Florida, who has worked for many years on the practical problems of germinating orchid seeds, has supplied all the seeds used in these experiments. He has likewise supplied certain information on the practical difficulties and on

various results obtained by him. For all of these favors and for constant interest I wish to express my thanks.

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LITERATURE CITED

1. BACHMANN, F. M., Vitamine requirements of certain yeasts. Jour. Biol. Chem. **39**:235. 1919.
2. BERNARD, NOEL, L'évolution dans la symbiose, les Orchidées et leur champignons commensaux. Ann. Sci. Nat. Bot. **9**:1–196. 1909.
3. BOTTOMLEY, W. B., The effect of nitrogen-fixing organisms and nucleic acid derivatives on plant growth. Roy. Soc. London Proc. B **91**:83–95. 1919.
4. BURGEFF, HANS, Die Wurzelpilze der Orchideen, ihre Kultur und ihre Leben in der Pflanze. Jena. 1909.
5. DUGGAR, B. M., SEVERY, J. W., and SCHMITZ, H., Studies in the physiology of fungi. IV. The growth of certain fungi in plant decoctions. Ann. Mo. Bot. Gard. **4**:165–173. 1917.
6. KING, JOHN, King's American Dispensatory, re-written and enlarged by H. W. FELTER and J. W. LLOYD, 18th ed., 3d revision, **2**:1898 (p. 1699).
7. KNUDSON, L., Influence of certain carbohydrates on green plants. Cornell Univ. Agric. Exp. Sta. Memoir **9**:1–75. 1916.
8. KNUDSON, L., and LINDSTROM, E. W., Influence of sugars on the growth of albino plants. Amer. Jour. Bot. **6**:401–405. 1919.
9. MAZÉ, P., and PERRIER, A., Recherches sur l'assimilation de quelques substances ternaires par les végétaux à chlorophylle. Inst. Pasteur Ann. **18**:721–747. 1904.
10. SERVETTAZ, CAMILLE, Recherches expérimentales sur la développement et la nutrition des mousses en milieux stérilisés. Ann. Sci. Nat. Bot. IX. **17**:111–224. 1913.
11. WILLAMAN, J. J., The function of vitamines in the metabolism of *Sclerotinia cinerea*. Jour. Amer. Chem. Soc. **42**:549–585. 1920.
12. WILLIAMS, The vitamine requirements of yeasts. Jour. Biol. Chem. **38**:465–486. 1919.
13. WILSON, J. K., Calcium hypochlorite as a seed sterilizer. Amer. Jour. Bot. **2**:420–427. 1915.